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L1 cell adhesion molecule induces melanoma cell motility by activation of mitogen-activated protein kinase pathways

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L1 cell adhesion molecule (L1CAM) is highly expressed in various types of cancer cells and has been implicated in the control of cell proliferation and motility. Recently, L1CAM was reported to induce the motility of melanoma cells, but the mechanism of this induction remains poorly understood. In this study, we investigated the molecular mechanisms by which L1CAM induces the motility of melanoma cells. Unlike other types of cancer cells, B16F10 melanoma cells highly expressed L1CAM at both the RNA and protein levels, and the expression of L1CAM induced AP-1 activity. In accordance to AP-1 activation, MAPK signaling pathways were activated by L1CAM. Inhibition of L1CAM expression by L1CAM-specific siRNA suppressed the activation of MAPKs such as ERK and p38. However, no significant change was observed in JNK activation. As expected, upstream MAP2K, MKK3/6, MAP3K, and TAK1 were also deactivated by the inhibition of L1CAM expression. L1CAM induced the motility of B16F10 cells. Inhibition of L1CAM expression suppressed migration and invasion of B16F10 cells, but no suppressive effect was observed on their proliferation and anti-apoptotic resistance. Treatment of B16F10 cells with U0126, an ERK inhibitor, or SB203580, a p38 inhibitor, suppressed the migration and invasion abilities of B16F10 cells. Taken together, our results suggest that L1CAM induces the motility of B16F10 melanoma cells *via* the activation of MAPK pathways. This finding provides a more detailed molecular mechanism of L1CAM-mediated induction of melanoma cell motility.

1. Introduction

L1 cell adhesion molecule (L1CAM) is a transmembrane glycoprotein with molecular weight of 200 - 220 kDa which belongs to an immunoglobulin superfamily. It consists of three parts: an ectodomain containing six immunoglobulin (Ig)-like domains (Ig1-Ig6) and five fibronectin (Fn)-type III homologous repeats (Fn1-Fn5), a short, single-pass transmembrane domain, and an intracellular domain (Moos et al. 1988). L1CAM was initially identified as a critical player in the development of the nervous system, including cell-cell interactions, neuronal cell migration, myelination, axonal outgrowth, and fasciculation (Brummendorf and Rathjen 1995; Kaifi et al. 2006; Rathjen and Schachner 1984). The ectodomain of L1CAM, and the Ig-like domains in particular are engaged in interactions with multiple proteins, including neural Ig CAM family proteins (Kuhn et al. 1991; Lustig et al. 1999; Moos et al. 1988; Silletti et al. 2000), non-Ig CAM family proteins (Blaess et al. 1998; Felding-Habermann et al. 1997; Oleszewski et al. 1999; Silletti et al. 2000), extracellular matrix proteins (Grumet et al. 1993), and signaling receptors (Castellani et al. 2002). These homophilic and heterophilic interactions of L1CAM ectodomain on the cell surface initiate signal transduction and mediate critical functions in neuronal cells, such as axonal guidance, development of the nervous system, and cell migration (Maness and Schachner 2007).

Recent studies have reported that L1CAM is aberrantly expressed in various types of cancers, including ovarian and uterine carcinomas, colon carcinomas, malignant gliomas, neuroblastomas, melanomas, renal cell carcinomas, cholangiocarcinomas, and gallbladder carcinoma, and the expression level of L1CAM correlates with the stages of tumor progression (Choi et al. 2011; Li et al. 2009; Raveh et al. 2009). Many studies have reported the roles of L1CAM in tumor progression. Expression of L1CAM in carcinoma cells increases cell motility during migration and invasion, as well as tumorigenesis (Gast et al. 2005; Gavert et al. 2005; Min et al. 2010; Silletti et al. 2004). The ectcodomain of L1CAM is released from the cell surface by proteolytic cleavage and binds to cell surface integrins in an autocrine or paracrine manner, increasing the motility and cell survival of cancer cells (Duczmal et al. 1997; Gutwein et al. 2003; Mechtersheimer et al. 2001; Voura et al. 2001).

Melanoma is an aggressive and potentially fatal form of skin cancer that arises from melanocytes. Several studies have reported a relationship between L1CAM and melanoma. L1CAM is expressed in melanoma tissues and several types of melanoma cells at different stages, and the expression of L1CAM correlates with melanoma progression (Meier et al. 2006). L1CAM is known to activate signaling molecules such as focal adhesion kinase (FAK), AKT (Min et al. 2010), ERK (Silletti et al. 2004), c-Src (Schmid and Maness 2008), phosphoinositide 3-kinase (PI3K) (Thelen et al. 2002), and Rac (Schmid et al. 2004) in different types of cancers. L1CAM expressed in melanoma cells has also been shown to activate ERK (Meier et al. 2006). ERK activation by L1CAM has also been previously reported in several other types of cancers, but the molecular mechanism whereby L1CAM induces cancer progression

remains poorly understood. Here we examined the molecular mechanism of L1CAM-activated induction of melanoma cell motility. We found that, besides ERK, p38MAPK was activated in the L1CAM signaling pathway, and p38MAPK induced L1CAM-mediated induction of melanoma cell migration and invasion. Moreover, upstream MAPK molecules, such as MKK3/6 (MAP2K) and TAK1 (MAP3K) were also activated in this process.

2. Investigations, results and discussion

2.1. L1CAM was highly expressed in B16F10 melanoma cells and induced AP-1 activity

Prior to investigation of L1CAM function, we examined expression of L1CAM on various types of cells. L1CAM protein and RNA expression levels were confirmed by FACS analyses and reverse transcriptase-PCR (RT-PCR), respectively. B16F10 melanoma cells expressed high levels of both L1CAM protein (Fig. 1A) and RNA (Fig. 1B). This observation was expected since melanoma tissues and other types of melanoma cells have been previously reported to express L1CAM (Lee et al. 2010; Meier et al. 2006). MDA-MB-231 breast cancer cells also expressed high levels of both L1CAM protein (Fig. 1A) and RNA (Fig. 1B) in accordance with a previous report (Li and Galileo 2010). Huh7 hepatocellular carcinoma cells showed low expression of L1CAM protein and RNA (Fig. 1A, B). As expected (Lee et al. 2012, 2009; Min et al. 2010), SCK cholangiocarcinoma cells expressed high levels of L1CAM protein and RNA (Fig. 1A, B). HaCaT keratinocyte expressed L1CAM protein and RNA, as previously reported (Lee et al. 2010), but the expression levels were low (Fig. 1A, B). Raw264.7 cells showed a low but detectable level of L1CAM (Fig. 1A, B). L1CAM expression could not be detected in peritoneal macrophages isolated from mice (Fig. 1A, B).

Since L1CAM is known to induce motility in carcinoma cells through ERK activation (Silletti et al. 2004), we next examined whether L1CAM induced AP-1 signaling pathways in B16F10 cells, using a reporter gene assay. The expression of L1CAM in B16F10 cells was inhibited by L1CAM-specific siRNA(siL1) and AP-1 luciferase reporter gene activity was measured. Inhibition of L1CAM expression by siL1 was confirmed at the protein and RNA levels (Fig. 1C upper panel). We found that AP-1 luciferase reporter gene activity was suppressed in siL1-transfected B16F10 cells compared to control siRNA(SC)-transfected cells (Fig. 1C, lower panel). We also examined L1CAM-mediated activation of NF-κB signaling pathways by reporter gene assay, since L1CAM activates NF-κB signaling pathways (Kiefel et al. 2011). However, NF-κB luciferase reporter gene activity was not significantly decreased in siL1-transfected B16F10 cells compared to control SC-transfected cells (data not shown).

2.2. L1CAM-activated MAPK signaling pathways

Since L1CAM induced AP-1 luciferase reporter gene activity, we next examined whether L1CAM activated MAPKs in B16F10 cells. The phosphorylation levels of MAPKs in B16F10 cells transfected with either siL1 or control SC were examined by Western blot analyses (Fig. 2A). We found that phosphorylation levels of p38 and ERK were dramatically decreased in siL1-transfected B10F10 cells compared to control SC-transfected cells. However, the phosphorylation level of JNK was not significantly different between the two groups, implying that L1CAM activated AP-1 signaling pathways through the activation of p38 and ERK rather than JNK.

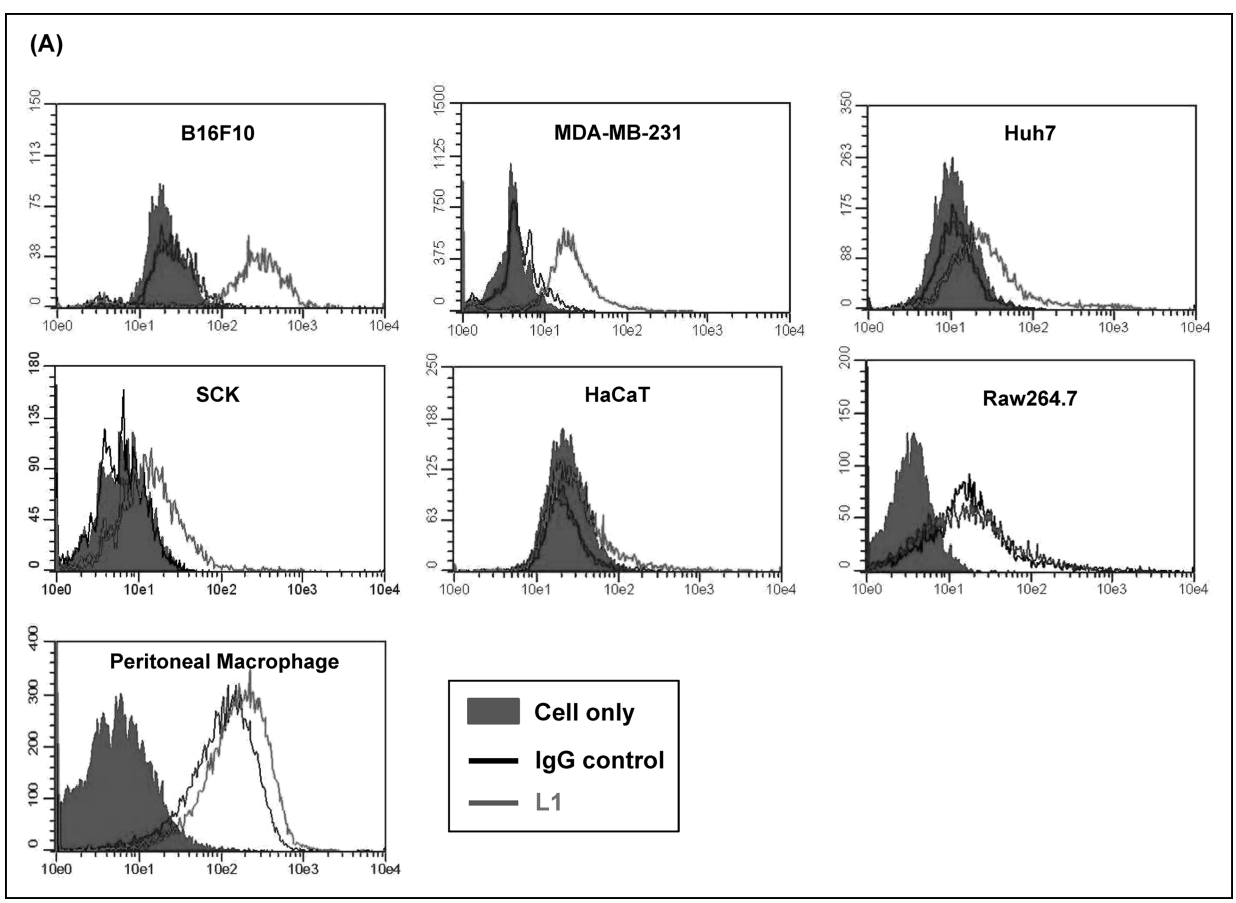

Fig. 1: *(Continued).*

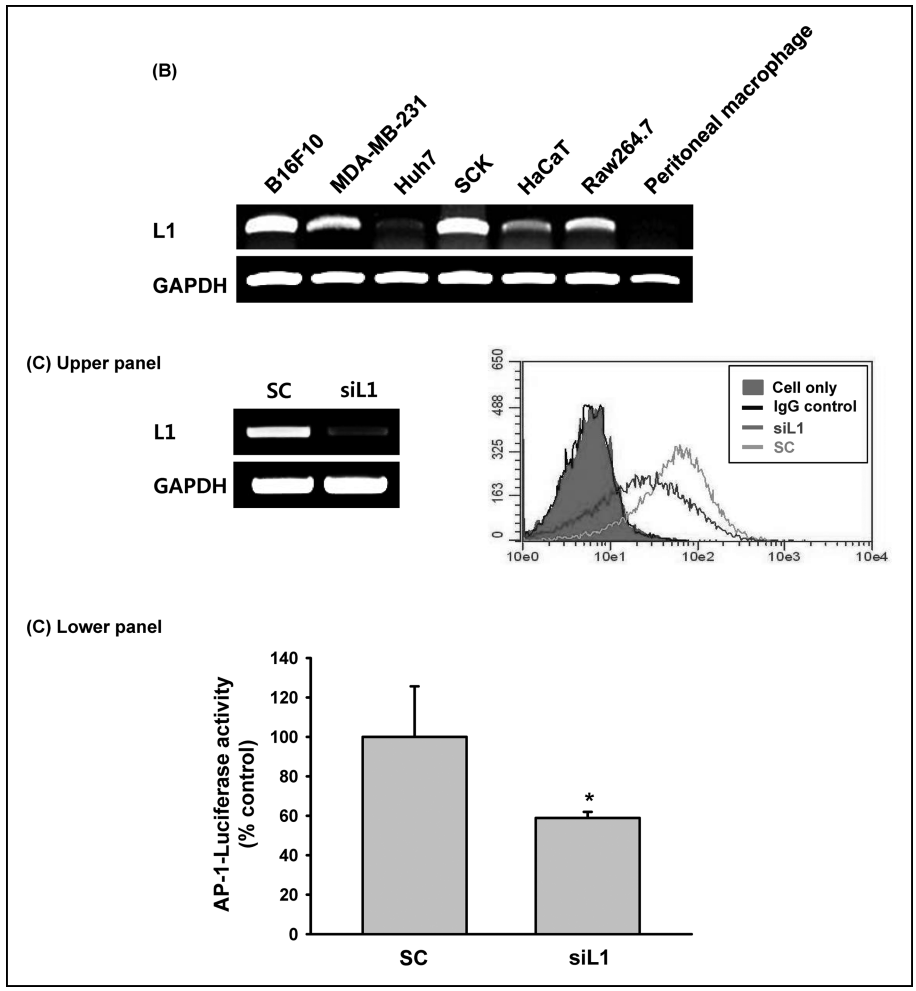

Fig. 1: High expression of L1CAM in B16F10 melanoma cells induces AP-1 activity. L1CAM expression at the (A) protein level and (B) RNA level in different types of cells (B16F10; melanoma, MDA-MB-231; breast cancer, Huh7; hepatocellular carcinoma, SCK; cholangiocarcinoma, HaCaT; keratinocyte, Raw264.7; macrophage-like cells, and peritoneal macrophages) was confirmed by FACS analyses and RT-PCR, respectively. (C; upper panel) Inhibition of L1 expression by siL1 at the RNA and protein levels in B16F10 cells was confirmed by RT-PCR and FACS analysis, respectively. (C; lower panel) B16F10 cells were co-transfected with AP-1-Luciferase reporter gene construct and β-galactosidase construct (transfection control), and luciferase activity was measured 48 h after transfection with a luminometer. $*P < 0.05$, compared to the control.

Next, we investigated whether L1CAM activated MAP2K and MAP3K, upstream activators of MAPKs, in B16F10 cells. The phosphorylation level of MKK3/6, an upstream MAP2K of p38 was dramatically decreased in siL1-transfected B16F10 cells compared to control SC-transfected cells. In contrast, the phosphorylation level of MEK1/2, an upstream MAP2K of ERK, was not significantly different between the two groups (Fig. 2B). Activation of MAP3K was also examined, and the TAK1 phosphorylation level was decreased in siL1-transfected B16F10 cells compared to control SC-transfected cells (Fig. 2C). Taken together, we found that L1CAM activated AP-1 signaling pathways through the activation of MAP3K, TAK1, MAP2K, MKK3/6 and MAPKs, p38 and ERK. p38 and its upstream MAP2K MKK3/6 were activated by L1CAM, but the MEK1/2, the MAP2K upstream of ERK, was not activated by L1CAM. These findings suggested that p38 may be activated by L1CAM through conventional MAP3K-MAP2K-MAPK pathways, whereas L1CAM-mediated ERK activation did not occur by conventional pathways and may involve unknown upstream activators of ERK, rather than MEK1.

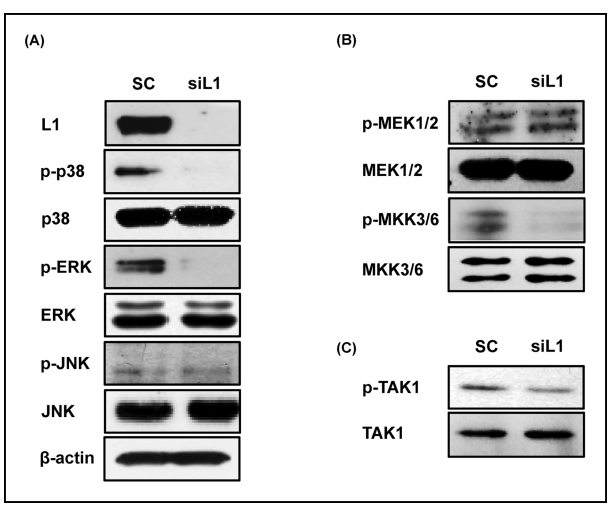

Fig. 2: L1CAM activates MAPKs pathways. B16F10 cells transfected with either siL1 or control SC were lysed, and the levels of phospho- and total forms of (A) MAPKs (p38, ERK, JNK), (B) MAP2Ks (MEK1/2, MKK3/6), and (C) MAP3K (TAK1) were identified by Western blot analyses.

2.3. L1CAM induced motility of B16F10 cells

Next, we examined the biological functions of L1CAM in B16F10 cells. Since L1CAM has been known to play a critical role in tumor cell motility (Kiefel et al. 2012), we first tested the role of L1CAM in cell migration. B16F10 cells were transfected with either siL1 or control SC followed by comparison of cell migration ability. As shown in Fig. 3A, migration ability was suppressed in siL1-transfected B16F10 cells compared to

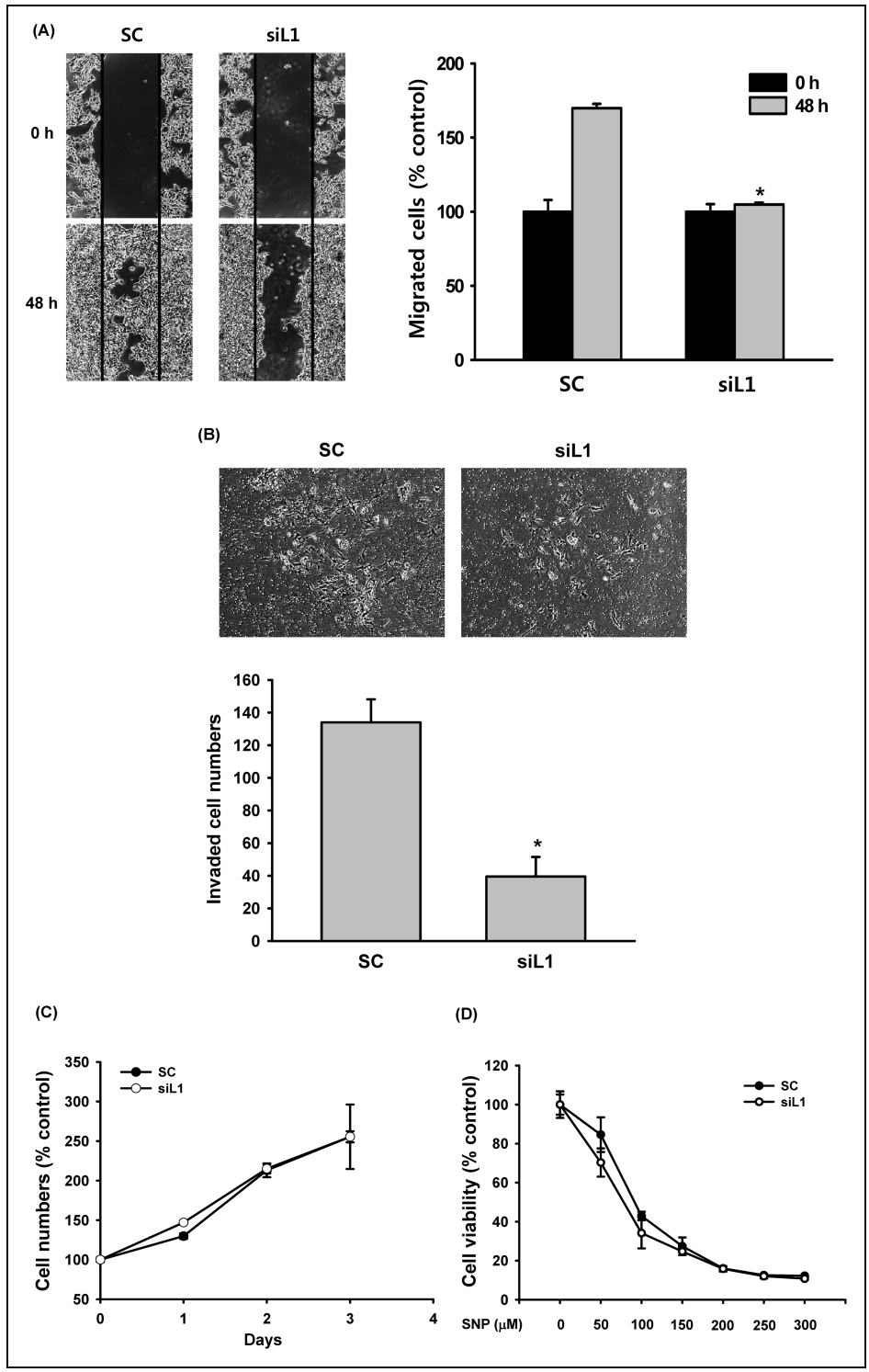

Fig. 3: L1CAM induces the migration and invasion of B16F10 melanoma cells. (A) B16F10 cells were transfected with either siL1 or control SC for 48 h. The cells were scratched and incubated for an additional 48 h. The migrated cells were photographed under a light microscope and quantified by counting. (B) B16F10 cells transfected with either siL1 or control SC were subjected to Matrigel invasion assay. The invaded cells were photographed under a light microscope and quantified by counting. (C) B16F10 cells transfected with either siL1 or control SC were incubated for 3 days before counting every 24 h with a hemocytometer. (D) B16F10 cells transfected with either siL1 or control SC were treated with SNP at the indicated concentrations for 24 h. Cell viability was measured by MTT assay. *$P < 0.05$, compared to the control.

control SC-transfected cells. We also tested the invasive ability of both groups of cells. As expected, siL1-transfected B16F10 cells showed reduced invasive ability compared to control SC-transfected cells (Fig. 3B), implying that L1CAM induced tumor cell motility, including migration and invasion, critical for cancer progression. To examine whether L1CAM had other roles in B16F10 cells, we tested whether L1CAM was involved in the induction of cell proliferation and anti-apoptotic ability in B16F10 cells. B16F10 cells were transfected with either siL1 or control SC, and cells were counted every 24 h. As shown in Fig. 3C, L1CAM had no significant effect on cell proliferation in both groups of B16F10 cells. The potential anti-apoptotic role of L1CAM in these cells was tested next using the well-known apoptosis-inducing agent sodium nitroprusside (SNP) (Chae et al. 2001; Pan et al. 2004; Yamada et al. 1996). As shown in Fig. 3D, L1CAM had no significant effect on SNP-induced apoptosis of B16F10 cells in both groups. Taken together, these data suggested that L1CAM induced tumor cell motility, includ-

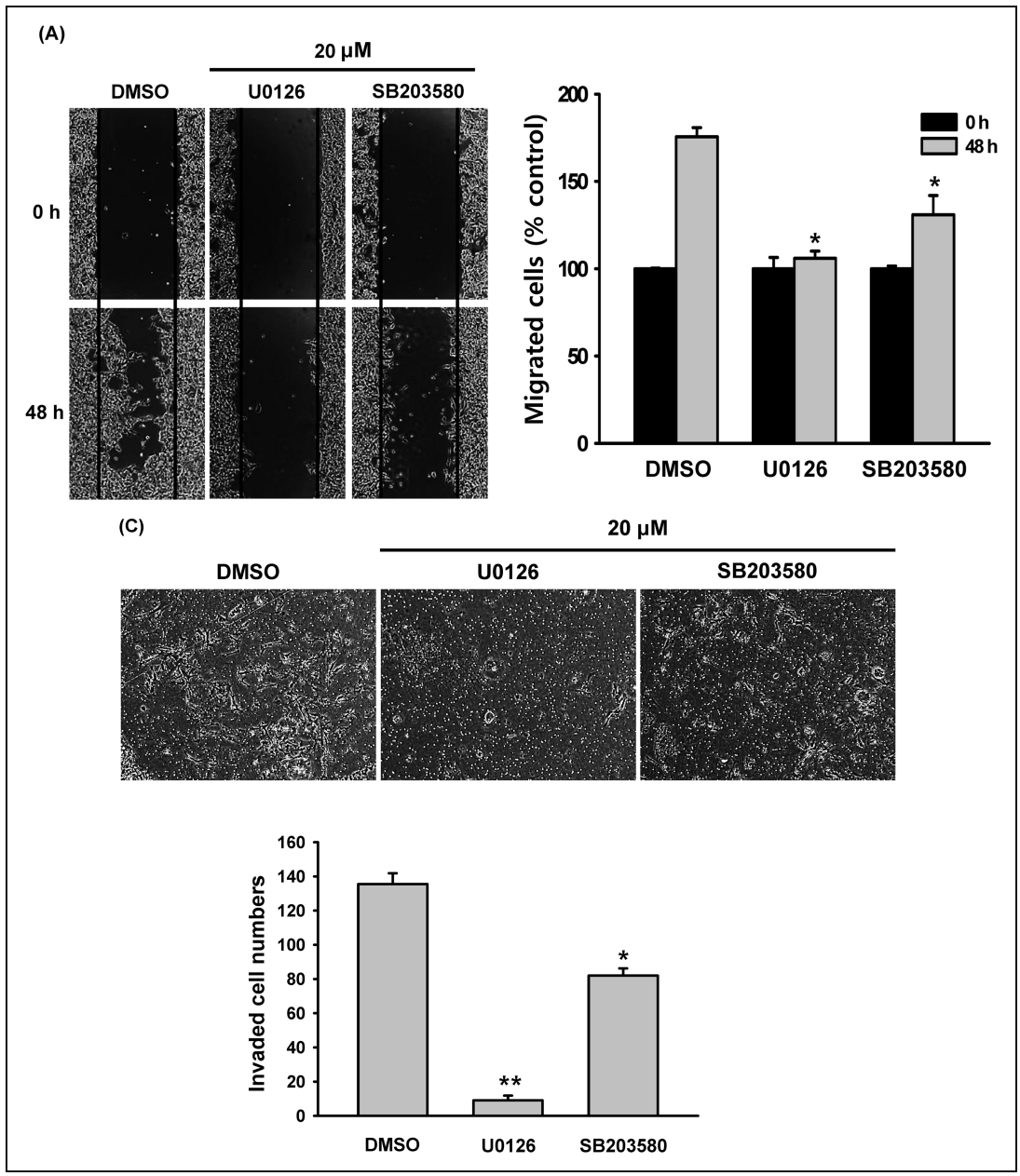

Fig. 4: L1CAM induces the migration and invasion of B16F10 melanoma cells through the activation of MAPKs. (A) B16F10 cells were plated for 24 h and then scratched. The cells were incubated for a further 48 h in the presence of either DMSO, U0126 (ERK inhibitor) or SB203580 (p38 inhibitor). The migrated cells were photographed under a light microscope and quantified by counting. (B) B16F10 cells in the presence of either DMSO, U0126 (ERK inhibitor) or SB203580 (p38 inhibitor) were subjected to Matrigel invasion assay. The invaded cells were photographed under a light microscope and quantified by counting. $*P < 0.05$, $**P < 0.01$, compared to the control.

ing cell migration and invasion rather than cell proliferation, and anti-apoptosis in B16F10 cells. This observation was consistent with previous studies which suggested the main function of L1CAM is to induce tumor cell motility.

2.4. L1CAM induced B16F10 cell motility through the activation of MAPKs

Since L1CAM activated MAPK signaling pathways (Fig. 2) and induced cell motility (Fig. 3), we wanted to investigate whether L1CAM-induced cell motility was mediated by the activation of MAPKs. We thus tested cell motility in the presence of MAPK-specific inhibitors. The migration abilities of siL1-transfected or control SC-transfected B16F10 cells were tested in the presence or absence of either U0126, an ERK inhibitor, or SB203580, a p38 inhibitor. As shown in Fig. 4A, the migration abilities of U0126- or SB203580-treated B16F10 cells were significantly suppressed compared to that of vehicle-treated B16F10 cells. The invasive abilities of siL1-transfected or control siRNA-transfected B16F10 cells were also tested in the presence or absence of either U0126 or SB203580. As shown in Fig. 4B, the invasive abilities of U0126- or SB203580-treated B16F10 cells were also suppressed compared to that of vehicle-treated B16F10 cells. Interestingly, although both U0126 and SB203580 suppressed migration and invasion of B16F10 cells, U0126 showed a higher level of suppression than SB203580 on both migration and invasion of B16F10 cells, implying that ERK may be a more critical MAPK compared to p38 in L1CAM-mediated induction of B16F10 cell motility. In addition, whereas U0126 suppressed both migration and invasion of B16F10 cells to nearly the basal level, SB203580 suppressed migration and invasion ability by ~ 60% and ~40%, respectively. Thus ERK may be more involved in migration than invasion of B16F10 cells, and there other molecules may be participating more in invasion of B16F10 cells. These findings may therefore suggest

the existence of different molecular mechanisms for migration and invasion of B16F10 cells.

In conclusion, we demonstrated that L1CAM induced B16F10 cell motility, including migration and invasion, through the activation of MAPK signaling pathways. Our study provided a better understanding of the molecular mechanism of L1CAM-mediated induction of cancer progression. Although other molecules involved in this process need to be identified, and the role of AP-1-targeted genes in the induction of tumor cell motility remain to be elucidated, our study provided a more detailed knowledge of the molecular mechanisms of L1CAM-mediated induction of melanoma cell motility. Our results provide not only new insights for a better understanding of the mechanism of L1CAM-mediated cancer progression, but also additional targets for the development of anti-cancer agents for the treatment of melanoma.

3. Experimental

3.1. Cells and cell culture

B16F10, Huh7, HaCaT, MDA-MB-231, and SCK cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA). Raw264.7 cells were maintained in RPMI 1640 media (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and antibiotics (penicillin and streptomycin; Gibco, USA) at 37 °Cin a humidified incubator with 5% CO_2. Peritoneal macrophages were obtained from C57BL/6 mice (Daehan-Biolink, Korea) 4 days after intraperitoneal injection of 4% thioglycolate (Sigma, USA), and were maintained in RPMI 1640 media under the same incubation conditions.

3.2. Transfection of L1CAM siRNA

L1CAM siRNA (siL1) and control siRNA were synthesized by Genolution Inc. B16F10 cells were plated in antibiotic-free DMEM media for 24 h and transiently transfected with either siL1 or control siRNA using Lipofectamine® RNAiMAX reagent (*Invitrogen*, USA), according to the manufacturer's instructions. Forty-eight hours after transfection, the cells were used for analyses of L1CAM expression level, cell signaling, and various assays, including luciferase assay, migration assay, invasion assay, cell proliferation assay, and anti-apoptosis assay.

3.3. Flow cytometry analysis

Cells were incubated with anti-L1CAM antibody (kindly provided by Dr. HyoJeong Hong) in FACS buffer (PBS with 0.1% BSA and 0.01% sodium azide) for 30 min on ice. After washing cells three times with FACS buffer, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody in FACS buffer for 30 min on ice. After washing the cells three times with FACS buffer, cells were resuspended in PBS and antibody binding was analyzed with a flow cytometer (Millipore, Germany).

3.4. RT-PCR

Total RNA was extracted from the cells using TRI reagent® (Molecular Research Center, Inc., USA) according to the manufacturer's instructions and stored at -70 °C until use. cDNA was synthesized from 1 μg of total RNA using PCR premix (Bioneer, Korea) with target-specific primers. The primer sequences used for PCR and reaction parameters were as follows: L1CAM (forward primer: 5'-TACCGCTTCCAGCTTCAG-3'; reverse primer: 5'-TGATGAAGCAGAGGATGAGC-3'), denaturation at 95 °C for 5 min, 30 cycles of 95 °C for 1 min, annealing at 50 °C for 1 min, elongation at 72 °C for 30 s, and final extension at 72 °C for 5 min; GAPDH (forward primer: 5'-CACTCACGGCAAATTCAACGGCA-3'; reverse primer: 5'-GACTCCACGACATACTCAGCAC-3'), denaturation at 95 °C for 5 min, 30 cycles of 95 °C for 1 min, annealing at 58 °C for 1 min, and elongation at 72 °C for 30 s, and final extension at 72 °C for 5 min.

3.5. In vitro cell migration assay

B16F10 cells were grown to a confluent monolayer before transfection with either siL1 or control siRNA for 48 h or treatment with MAPK inhibitors (U0126 or SB203580). The cells were scratched with a pipette tip as previously described (Zhao et al. 2006). After 48 h, migrated cells were visualized using an Inverted Phase Microscope (Olympus, Japan) and migrated areas were quantified using a pixel counter.

3.6. In vitro invasion assay

The invasion assay was performed using a Matrigel (BD Biosciences, USA)-coated transwell (Corning Costa, USA), as previously described (Jung et al. 2013). Briefly, the upper surface of a transwell was coated with 25 μg of Matrigel and DMEM without FBS were placed in the lower well. B16F10 cells (1×10^5) transfected with either siL1 or control siRNA for 48 h or treated with MAPK inhibitors (U0126 or SB203580) were resuspended in 100 μl DMEM containing 10% FBS and loaded into each of the upper wells. After 48 h, the cells on the upper surface of the well were removed using a cotton swab and the cells on the lower surface of the well were fixed with 4% paraformaldehyde (Sigma, USA) for 1 h at 4 °C and stained with H&E. The invasive cells were photographed using an Inverted Phase Microscope (Olympus, Japan) and quantified by counting.

3.7. Cell proliferation assay

B16F10 cells transfected with either siL1 or control siRNA for 48 h were grown in DMEM for 3 days. Cell numbers of each group were determined daily using a hemocytometer.

3.8. Apoptosis assay

B16F10 cells transfected with either siL1 or control siRNA for 48 h were grown in DMEM containing various concentration of sodium nitroprusside (SNP). Cell viability was measured 24 h later, by conventional MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolum bromide] assay. The absorbance of samples was measured at 490 nm using a microplate reader (Molecular Devices Corp., USA).

3.9. Luciferase reporter gene assay

B16F10 cells were transfected with siL1 (or control siRNA), AP-1 luciferase construct and β-galactosidase construct using Lipofectamine 2000™ according to the manufacturer's instructions. Cells were lysed 48 h later with cell culture lysis reagent (Promega, USA) at 4 °C for 1 h. The cell lysate was mixed with the same volume of luciferin followed by measurement of luminescence with a luminometer (BioTek, USA). Transfection efficiency was normalized by X-gal reaction, and the absorbance was measured at 570 nm with a fluorescence spectrophotometer (BioTek, USA).

3.10. Preparation of whole cell lysate, nuclear fraction and Western blot analysis

For whole cell lysate preparation, B16F10 cells were lysed using RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) for 30 min in ice followed by sonication (Thermo Fischer Scientific, USA) for 10 sec.

For nuclear fraction preparation, B16F10 cells were lysed using buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.05% NP-40, pH 7.9) for 10 min on ice and centrifuged at 3,000 rpm for 10 min at 4 °C. The supernatant was removed and the pellet was resuspended in buffer B (5 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol (v/v), 300 mM NaCl, pH 7.9) and sonicated for 10 s on ice. The lysate was centrifuged at 24,000g for 20 min at 4 °C and the supernatant was collected as the nuclear fraction. Protein concentrations were quantified in all samples by the Bradford assay method, and samples were stored at -20 °C until use. For Western blot analysis, whole cell lysates or nuclear fractions were subjected to SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA). Target proteins were detected with primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies, and immune complexes were visualized with the enhanced chemiluminescence system (AbFrontier, Korea)

3.11. Statistical analysis

All data are presented as the mean ± standard deviation (SD). Statistical comparisons between groups were performed by one-way ANOVA and Student's t-test. $*P$ value < 0.05 and $**P$ value < 0.01 were considered statistically significant.

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